

# Tumor Biomarker Glycoproteins in the Seminal Plasma of Healthy Human Males Are Endogenous Ligands for DC-SIGN<sup>§</sup>

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DC-SIGN is an immune C-type lectin that is expressed on both immature and mature dendritic cells associated with peripheral and lymphoid tissues in humans. It is a pattern recognition receptor that binds to several pathogens including HIV-1, Ebola virus, *Mycobacterium tuberculosis*, *Candida albicans*, *Helicobacter pylori*, and *Schistosoma mansoni*. Evidence is now mounting that DC-SIGN also recognizes endogenous glycoproteins, and that such interactions play a major role in maintaining immune homeostasis in humans and mice. Autoantigens (neoantigens) are produced for the first time in the human testes and other organs of the male urogenital tract under androgenic stimulus during puberty. Such antigens trigger autoimmune orchitis if the immune response is not tightly regulated within this system. Endogenous ligands for DC-SIGN could play a role in modulating such responses. Human seminal plasma glycoproteins express a high level of terminal Lewis<sup>x</sup> and Lewis<sup>y</sup> carbohydrate antigens. These epitopes react specifically with the lectin domains of DC-SIGN. However, because the expression of these sequences is necessary but not sufficient for interaction with DC-SIGN, this study was undertaken to determine if any seminal plasma glycoproteins are also endogenous ligands for DC-SIGN. Glycoproteins bearing terminal Lewis<sup>x</sup> and Lewis<sup>y</sup> sequences were initially isolated by lectin affinity chromatography. Protein sequencing established that three tumor biomarker glycoproteins (clusterin, galectin-3 binding glycoprotein, prostatic acid phosphatase) and protein C inhibitor were purified by using this affinity method. The binding of DC-SIGN to these seminal plasma glycoproteins was demonstrated in both

Western blot and immunoprecipitation studies. These findings have confirmed that human seminal plasma contains endogenous glycoprotein ligands for DC-SIGN that could play a role in maintaining immune homeostasis both in the male urogenital tract and the vagina after coitus. *Molecular & Cellular Proteomics* 11: 10.1074/mcp.M111.008730, 1–12, 2012.

Human C-type lectins are associated with a wide variety of different immune cell types and act as pattern-recognition receptors that play an essential role in the detection of many different pathogens that induce innate immune responses (1). Prominent among this repertoire of receptors is DC-SIGN<sup>1</sup> (2, 3). DC-SIGN binds to high mannose type N-glycans and several Lewis type carbohydrate sequences (Lewis<sup>a</sup>, Lewis<sup>b</sup>, Lewis<sup>x</sup>, Lewis<sup>y</sup>, sulfo-Lewis<sup>a</sup>, and pseudo-Lewis<sup>y</sup> antigens) (Table I) (4, 5).

Though these Lewis type sequences are expressed on many human glycoproteins, only a select subset has been reported to bind to DC-SIGN. They include bile-salt stimulated lipase (6), butyrophilin (7), carcinoembryonic antigen (8), carcinoma embryonic antigen related cell adhesion molecule, (9), ICAM-2 (10, 11), ICAM-3 (12), and Mac-1 (13). These findings indicate that a very specific orientation and/or density of Lewis type sequences on a glycoconjugate could enable DC-SIGN binding, and not simply the presence or absence of specific carbohydrate epitopes (1).

Lewis<sup>x</sup> and Lewis<sup>y</sup> sequences are profusely expressed in the human male urogenital tract. The results of an early study confirmed that N-glycans bearing these sequences were linked to prostate specific antigen (PSA), also known as  $\gamma$ -seminoprotein, kallikrein-3 or semenogelase (14). Further analysis of seminal plasma glycoproteins and mucins revealed that O-glycans terminated with Lewis<sup>x</sup> and Lewis<sup>y</sup> sequences were linked only to high MW mucins (>500 kDa)

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<sup>1</sup> The abbreviations used are: DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; PAP, prostatic acid phosphatase; PCI, protein C inhibitor; PSA, prostate specific antigen.

(15). Free oligosaccharides capped with these sequences were also present in human seminal plasma (16). More recent ultrasensitive mass spectrometric analyses have confirmed the prolific expression of N-glycans bearing antennae terminated with multiple Lewis<sup>x</sup> and Lewis<sup>y</sup> epitopes on glycoproteins associated with human sperm and seminal plasma (17, 18). These observations indicated that there could be many endogenous glycoprotein ligands for DC-SIGN in the male reproductive system. The current study was undertaken to identify the major seminal plasma glycoproteins that bind to this immune lectin.

#### EXPERIMENTAL PROCEDURES

**Materials**—*Lotus tetragonolobus* seeds were purchased from B&T World Seeds (Aigues-Vives, France). The lectin associated with these seeds was isolated by employing the same procedure developed previously by Smith and coworkers (19). *Lotus* lectin-agarose was generated by coupling freshly prepared lectin to Affi-Gel 15 (BioRad, Richmond, CA) at a final concentration of 26 mg/ml of packed gel. Amicon Ultra cartridges were purchased from Millipore (Carrington, Co. Cork, Ireland). Protease inhibitor mixture set III (EDTA free) and all glycosidases except bovine testes  $\beta$ -galactosidase were purchased from CalBiochem (Gibbstown, NJ). Dulbecco's phosphate buffered saline (PBS) was obtained from Invitrogen (Carlsbad, CA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) except as noted.

**Patient Participation**—This study was approved by the Health Sciences Institutional Review Board of the University of Missouri-Columbia. All research procedures involving materials originating from patients were conducted following the approved study protocol. Semen was obtained from fertile individuals who had fathered a child during the previous two years. All the specimens used in this study also had normal semen parameters based on World Health Organization (WHO) guidelines (20).

**Collection of Human Seminal Plasma**—Each semen sample was allowed to liquefy for 30 min at room temperature, diluted with an equal volume of phosphate-buffered saline (PBS), and centrifuged at  $600 \times g$  for 5 min. The supernatant was collected and centrifuged at  $2000 \times g$  for 5 min. The acellular supernatants containing diluted seminal plasma were collected individually and stored frozen at  $-20^{\circ}\text{C}$ .

**Affinity Chromatography on Lotus Lectin-Agarose**—This separation was carried out essentially as described previously (19), with some modifications. Seminal plasma samples were thawed under running tap water and centrifuged at  $18,000 \times g$  at  $4^{\circ}\text{C}$  for 30 min to remove any insoluble precipitate. The supernatants were harvested and supplemented with protease inhibitors according to the manufacturer's instructions. This fraction was adjusted to a Triton-X-100 concentration of 0.3% (v/v) by the addition of a 5% aqueous solution of this detergent in PBS. The detergent solubilized fraction was incubated at  $37^{\circ}\text{C}$  for 30 min. A small column containing 1 ml packed volume of *Lotus* lectin-agarose was equilibrated in PBS containing 0.3% Triton-X-100. A solubilized seminal plasma sample (2 ml) was applied to this column and eluted under gravity. The flow through was collected and reapplied to the column. After elution, this process was repeated again. The column was eluted under gravity, and fractions (2 ml) were collected and monitored at 280 nm until the absorbance dropped below 0.01. The column was stopped, and the matrix was eluted with 1 ml of PBS containing 0.1 M fucose. The eluant was collected, and the column was capped and incubated for 30 min. The column was opened, and 2 one ml aliquots of buffer containing 0.1 M fucose were applied to the column and collected separately. The column was

eluted with PBS, collecting 1-ml fractions. Fractions were analyzed by absorbance at 280 nm to detect the eluted glycoproteins. Fractions containing the bound glycoproteins were pooled, concentrated on Ultrafree cartridges against PBS to remove fucose, and stored frozen at  $-20^{\circ}\text{C}$  until analyzed.

**Identification of the Major Lotus Lectin Bound Glycoproteins**—Proteins from individual fractions eluted from *Lotus* lectin-agarose were precipitated in 10% trichloroacetic acid and analyzed by SDS-gel electrophoresis as described previously (21). To perform proteomic analysis of the Coomassie blue stained bands, they were excised, extracted with acetonitrile to remove the stain, and dried in a vacuum centrifuge. Proteins were reduced with 10 mM dithiothreitol, alkylated with 55 mM iodoacetic acid, digested with 20  $\mu\text{l}$  of trypsin working solution (Promega sequencing grade modified trypsin, prepared according to manufacturer's instructions, Promega, Madison, WI) and incubated overnight at  $37^{\circ}\text{C}$ . Peptides were released from gel slices by incubation with 0.1% trifluoroacetic acid for 10 min, followed by the addition of acetonitrile for 15 min at  $37^{\circ}\text{C}$ . This extraction process was repeated once more. Tryptic peptides were then dried on a vacuum centrifuge and resuspended in 80  $\mu\text{l}$  0.1% trifluoroacetic acid prior to mass spectrometry.

For offline liquid chromatography matrix-assisted laser desorption ionization/time of flight (MALDI TOF/TOF)-MS analysis, tryptic peptides were separated by using the Ultimate 3000 LC system (Dionex, Sunnyvale CA), fitted with a Pepmap analytical C-18 nanocapillary (75  $\mu\text{m}$  internal diameter  $\times$  15 cm length; Dionex). The digest was loaded onto the column and eluted using solvent A (0.1% (v/v) formic acid in 2% (v/v) acetonitrile) and solvent B (0.1% (v/v) formic acid in 90% (v/v) acetonitrile), in the following gradient: 0–60% solvent B (0–60 min), 60–90% solvent B (60–61 min), 90% solvent B (61–66 min) and 100% solvent A (66–67 min). Eluting fractions were mixed with  $\alpha$ -cyano-4-hydroxy cinnamic acid matrix and spotted onto a metal MALDI target plate using a Probot (LC Packings, Dionex, Sunnyvale, CA). Peptides were analyzed by MALDI-MS and MS-MS profiling on an Applied Biosystems 4800 MALDI-TOF/TOF mass spectrometer. The ten most abundant ions in each sample were sequenced. Peak picking was conducted using GPS Explorer software version 3.6 (Applied Biosystems, Foster City, CA). A signal-to-noise threshold of 10 was used. Sequazyme peptide mass standards were used as external standards for calibration purposes and no contaminant ions were excluded. For MS/MS experiments, peak list generation and database searching were conducted using GPS Explorer software version 3.6 (Applied Biosystems) with the default parameters. Both LC-MS and LC-MS/MS data were used to search 283,454 entries in release 54.2 of the SwissProt database with version 2.2 of the Mascot database search algorithm (www.matrixscience.com). The following parameters were implemented for this analysis: (1) peptide masses were fixed as monoisotopic; (2) partial oxidation of methionine residues was considered; (3) partial carboxymethylation of cysteine residues was considered; (4) the mass tolerance was set at 75 ppm for precursor ions and 0.1 Da for fragment ions; and (5) tryptic digests were assumed to have no more than one missed cleavage. Peptide matches from both MS and MS/MS data were used to generate probability-based Mowse protein scores. Scores greater than 55 were considered significant ( $p < 0.05$ ) (22). The search for other potential proteins in the *Lotus* lectin-binding fractions from seminal plasma was broadened by increasing the mass tolerance for precursor ions to 100 ppm, enlarging the taxonomy to include all species, and allowing the possibility of two missed cleavages.

**Processing of Seminal Plasma Glycoprotein Samples to Obtain N-Glycans**—Each sample was subjected to reduction, carboxymethylation, and tryptic digestion as described previously (23). Peptide N-glycosidase F digestion of the tryptic glycopeptides was carried out in 50 mM ammonium bicarbonate, pH 8.5, for 24 h at  $37^{\circ}\text{C}$  with 5

units of enzyme (Roche Applied Science, UK). The released *N*-glycans were purified from O-glycopeptides and peptides by chromatography on a Sep-Pak C18 cartridge (Waters Corp., Milford, MA) as described previously (24). The purified native *N*-glycans were either subsequently derivatized or subjected to modifications before derivatization. They were subjected to the sodium hydroxide permethylation procedure as described previously before mass spectrometric analyses (24).

**MS and MS/MS Analyses of Permethyated Seminal Plasma Glycans**—MALDI-TOF data were acquired on a Voyager-DE STR mass spectrometer (Applied Biosystems, Foster City, CA) in the reflectron mode with delayed extraction. Permethyated samples were dissolved in 10  $\mu$ l of 70% (v/v) aqueous methanol, and 1  $\mu$ l of dissolved sample was premixed with 1  $\mu$ l of matrix (20 mg/ml 2,5-dihydroxybenzoic acid in 70% (v/v) aqueous methanol), spotted onto a target plate, and dried under vacuum.

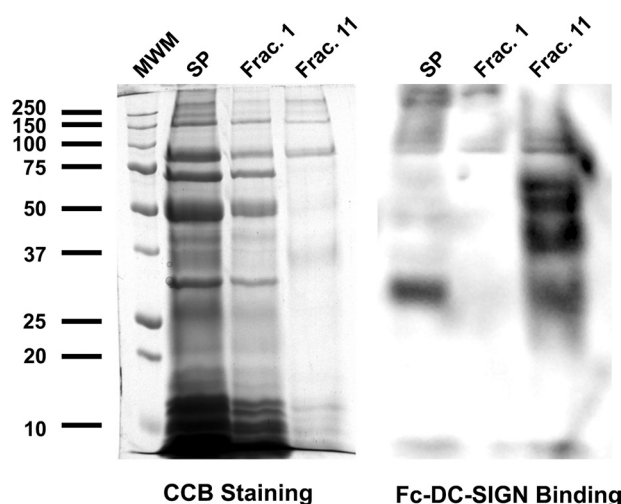
Further MS/MS analyses of peaks observed in the MS spectra were carried out by using a 4800 MALDI-TOF/TOF (Applied Biosystems) mass spectrometer in positive ion mode ( $M + Na$ )<sup>+</sup>. The collision energy was set to 1 kV, and argon was used as collision gas. Samples were dissolved in 10  $\mu$ l of methanol, and 1  $\mu$ l was mixed at a 1:1 ratio (v/v) with 2,5-dihydroxybenzoic acid (20 mg/ml in 70% methanol in water) as matrix.

**Analyses of MALDI Data**—The MS and MS/MS data were processed by using Data Explorer 4.9 Software (Applied Biosystems, UK). The mass spectra were baseline corrected (default settings), noise filtered (with correction factor of 0.7), and then converted to ASCII format. The processed spectra were subjected to manual assignment and annotation with the aid of a glycoinformatics tool known as GlycoWorkBench (25). Peak picking was done manually, and proposed assignments for the selected peaks were based on molecular mass composition of the <sup>12</sup>C isotope together with knowledge of the biosynthetic pathways. The proposed structures were then confirmed by data obtained from MS/MS experiments.

**Western blot analysis with DC-SIGN-Fc**—DC-SIGN-Fc consists of the extracellular portion of DC-SIGN (amino acid residues 64–404) fused at the COOH terminus to a human IgG1-Fc fragment (R&D Systems, Minneapolis, MN). The soluble DC-SIGN binding assay was performed as follows. Total seminal plasma proteins or fractions derived from the *Lotus* lectin-agarose column as indicated (~20  $\mu$ g total protein for each) were resolved by SDS-gel electrophoresis and transferred to Immobilon-P PVDF membranes as described previously (21, 26). Following transfer, PVDF membranes were rinsed three times with Dulbecco's PBS (DPBS) and then blocked for 30 min in StartingBlock Blocking Buffer (Thermo Fisher Scientific, Rockford, IL) at 23°C. Soluble DC-SIGN-Fc was diluted in StartingBlock Blocking Buffer to a final concentration of 15 nM and incubated with membranes for 2 h at RT. Unbound DC-SIGN-Fc was removed by washing membranes thrice with Dulbecco's PBS for 5 min per wash. Membranes were then incubated with a goat anti-human IgG-HRP conjugate for 15 min, washed three times as described above, and incubated with chemiluminescent substrate for 5 min (SuperSignal West Pico, Thermo Fisher Scientific, Rockford, IL). Images were captured by using a Cell Biosciences Alphamager system.

**Co-purification of soluble DC-SIGN-Fc with seminal plasma proteins**—The following antibodies were used in this study as indicated below: Goat anti-human galectin3-binding protein (1:500, R&D Systems, Minneapolis, MN), Mouse anti-human clusterin (1:250, Santa Cruz Biotechnology, Santa Cruz, CA), Mouse anti-human prostatic acid phosphatase (1:100, Santa Cruz Biotechnology), and rabbit anti-human protein C inhibitor (1:640, Abcam, Cambridge, MA).

Seminal plasma proteins derived from the *Lotus* lectin-agarose column (Fraction 11, ~50  $\mu$ g total protein) were diluted in 500  $\mu$ l of



**FIG. 1. Analysis of DC-SIGN binding to seminal plasma glycoproteins.** The seminal plasma fraction (10  $\mu$ l) was subjected to SDS gel electrophoresis and stained with colloidal Coomassie blue (CCB) as shown in the panel on the left. This sample was also subjected to Western blotting with the DC-SIGN-Fc (right panel). Aliquots (5  $\mu$ l) were also taken directly from fractions 1 (Frac. 1) and 11 (Frac. 11) obtained by chromatography on *Lotus* lectin-agarose (Fig. 2), and also subjected to both SDS gel electrophoresis and blotting with DC-SIGN-Fc.

DPBS and incubated overnight at 4°C either alone or in the presence of 3.25  $\mu$ g soluble DC-SIGN-Fc (Final concentration = 100 nM). Following incubation, samples were transferred to fresh tubes containing 20  $\mu$ l of protein-A Sepharose beads which had been previously equilibrated in DPBS. Samples were incubated for a further 60 min at 4°C with continuous rotation. Beads were collected by brief centrifugation (5000  $\times$  g, 1 min) and subjected to five washes with DPBS. Bound proteins were released from the beads by boiling in 40  $\mu$ l of Laemmli sample buffer, resolved by SDS-gel electrophoresis and transferred to Immobilon-P PVDF membranes as described previously (21, 26). Following transfer, PVDF membranes were rinsed three times with DPBS and then blocked for 30 min in 3% w/v nonfat milk in DPBS at 23°C. The indicated antibodies were diluted in 3% w/v nonfat milk in DPBS and incubated with membranes for 2 h at room temperature. Unbound antibody was removed by washing membranes three times with DPBS for 5 min per wash. Membranes were then incubated with appropriate secondary antibody-HRP conjugates for 30 min, washed three times as described above, and incubated with chemiluminescent substrate for 5 min (SuperSignal West Dura, Thermo Fisher Scientific, Rockford, IL). Images were captured using a Cell Biosciences Alphamager system.

## RESULTS

**Analysis of DC-SIGN Binding to Human Seminal Plasma Glycoproteins**—Human seminal plasma was isolated as previously described (17). This fraction was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1). The separated proteins were subjected to Western blot analysis with DC-SIGN-Fc. Only very weak binding of glycoproteins in the whole seminal plasma fraction was detected by using this approach, except for a minor band migrating at about 30–35 kDa (Fig. 1). This result indicated that there were very marginal amounts of glycoprotein ligands for DC-SIGN in human seminal plasma.



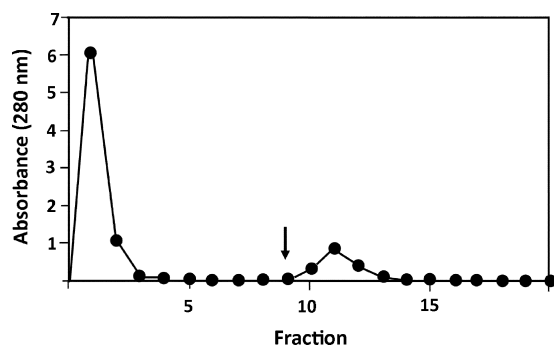


FIG. 2. Affinity chromatography of seminal plasma on *Lotus* lectin-agarose. Diluted seminal plasma was separated on this matrix as described in the methods. Elution with buffer containing 0.1 M fucose was initiated at the position indicated by the arrow.

**Affinity Chromatography of Seminal Plasma Proteins on *Lotus* Lectin-Agarose**—This low level of DC-SIGN-Fc binding during Western blot analysis was inconsistent with previous results indicating substantial modification of seminal plasma glycoproteins with Lewis<sup>x</sup> and Lewis<sup>y</sup> type sequences (17). Two potential reasons for this lack of binding were considered. One hypothesis was that the amount of DC-SIGN binding glycoproteins was substantially lower than expected based upon the glycomic analysis of human seminal plasma (17). Another possibility was that other proteins present in seminal plasma were interfering with the interaction of DC-SIGN with its glycoprotein ligands during Western blot analyses. One approach to evaluate both of these possibilities was to selectively enrich for seminal plasma glycoproteins that bear Lewis<sup>x</sup> and Lewis<sup>y</sup> sequences. *Lotus tetragonolobus* agglutinin is a lectin that readily binds to glycans terminated with these sequences (19, 27). Like DC-SIGN, this lectin also exists as a tetramer (5, 28). These properties indicated that it could potentially be useful for affinity purifying fucosylated endogenous glycoprotein ligands for DC-SIGN. A seminal plasma sample was diluted with detergent and protease inhibitors and applied to a small column (1 ml packed volume) of *Lotus* lectin-agarose. Bound glycoprotein was eluted with buffer containing 0.1 M fucose. A typical profile is shown in Fig. 2. The amount of protein bound to this *Lotus* lectin-agarose column averaged about 1 mg per separation based on optical density readings at 280 nm. Human seminal plasma contains about 50 mg/ml of protein (29). Therefore, the bound fraction represented only a very small proportion of the total protein in this fluid. No additional proteins were eluted by subsequent washing of the column with buffer containing 1 M fucose (data not shown). To confirm that this affinity column was not overloaded, the unbound fractions were pooled and subjected to another round of chromatography. Only a marginal amount of protein (<0.1 mg) was observed in the fractions that were eluted with fucose during this second round of chromatography. *Lotus* lectin-agarose was found to be stable under the conditions outlined in this study, generating highly reproducible results.

**Glycomics Analysis of Bound and Unbound Fractions Obtained by Affinity Chromatography on *Lotus* lectin-agarose**—The ability of this method to enrich for glycoproteins bearing terminal Lewis<sup>x</sup> and Lewis<sup>y</sup> sequences was determined by performing glycomic analysis of the seminal plasma fraction before and after affinity chromatography. The MALDI/MS spectrum of the N-glycans in the whole seminal plasma sample is shown in Fig. 3A. This fraction contained rather substantial amounts of high mannose type N-glycans (Man<sub>5–7</sub>GlcNAc<sub>2</sub>), as indicated by strong signals at *m/z* 1579, 1783, and 1987. Typical biantennary and triantennary N-glycans lacking sialic acid were observed as signals at *m/z* 2039, 2243, and 2693. The presence of sialylated complex type bi- and tetraantennary N-glycans was confirmed by signals at *m/z* 2431, 2604, and 2966. Biantennary N-glycans with 1–4 fucose residues were indicated by signals at *m/z* 2417, 2592, 2765, and 2940, respectively. Similarly, signals at *m/z* 2867, 3041, 3215, 3389, 3563, and 3737 indicated the presence of triantennary complex type N-glycans bearing 1–6 fucose residues. Low intensity signals at *m/z* 3839, 4013, 4187, 4361, and 4534 confirmed the presence of tetraantennary N-glycans bearing 4–8 fucose residues (Fig. 3A). The expression of such N-glycans in human seminal plasma was previously documented (17).

Glycomic analysis of the N-glycans derived from the glycoproteins present in the unbound and bound fractions after lectin affinity chromatography revealed some distinct differences compared with the total seminal plasma fraction (Figs. 3B, 3C). The signal intensities of glycans associated with the glycoproteins in the total and unbound fractions were essentially the same for the following classes of N-glycans: (1) high mannose; (2) sialylated biantennary N-glycans; and (3) biantennary type N-glycans expressing 0–4 fucose residues on their antennae (Figs. 3A, 3B; Fig. 4 (upper panel)). However, the relative intensities of the signals for the triantennary N-glycans bearing Lewis sequences were substantially diminished in the unbound glycoprotein fraction compared with the total fraction (Fig. 4 (middle panel)). This decrease in signal intensities of the triantennary N-glycans in the unbound fraction was correlated with a substantial increase in the signals associated with the bound fraction (Figs. 3B, 3C; Fig. 4 (middle panel)). Signals for tetraantennary N-glycans bearing 4–8 fucose residues on their antennae were detected in the total fraction but not in the unbound fraction (Fig. 4 (lower panel)). By contrast, the intensities of the signals for these fucosylated tetraantennary N-glycans were substantially enhanced in the bound fraction compared with the total fraction (Fig. 4 (lower panel)). Glycomic analysis of the N-glycans in the bound fraction indicated that the relative intensities of the signals for high mannose type N-glycans were substantially reduced compared with the total or unbound fractions (Fig. 4 (upper panel)). The intensity of the signals for the sialylated biantennary type N-glycans was also decreased in the bound fraction compared with the total or unbound fractions (Figs. 3B, 3C). This same pattern of enrichment and

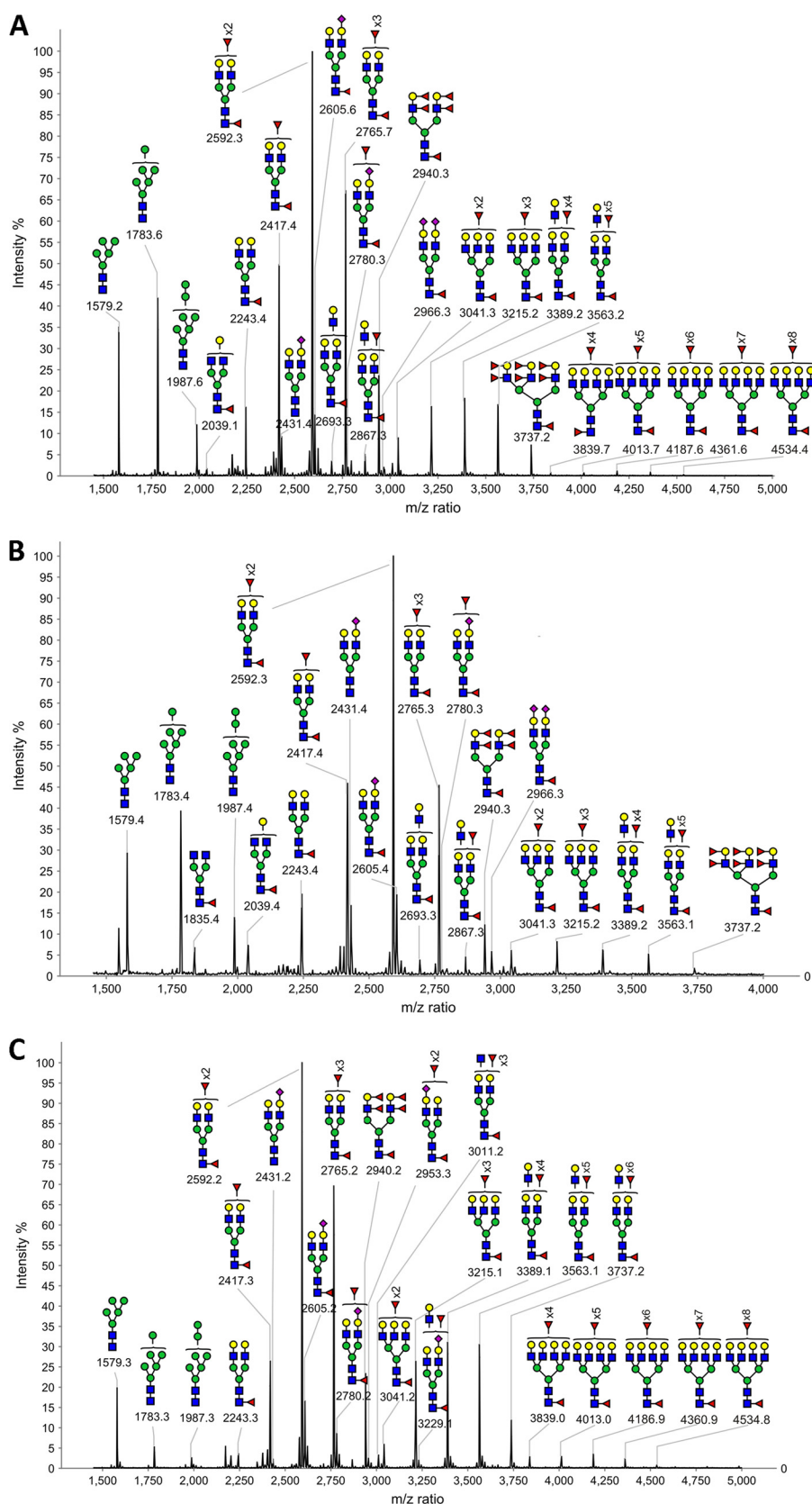


FIG. 3. MALDI-TOF profiling of N-glycans associated with glycoprotein fractions obtained by lectin affinity chromatography of human seminal plasma. The N-glycans associated with the glycoproteins in a whole human seminal plasma fraction before it was subjected to lectin affinity chromatography as shown in Fig. 2 were determined (panel A). The N-glycans associated with the pooled unbound (panel B) and bound fractions (panel C) obtained after affinity chromatography on *Lotus* lectin-agarose were also defined.

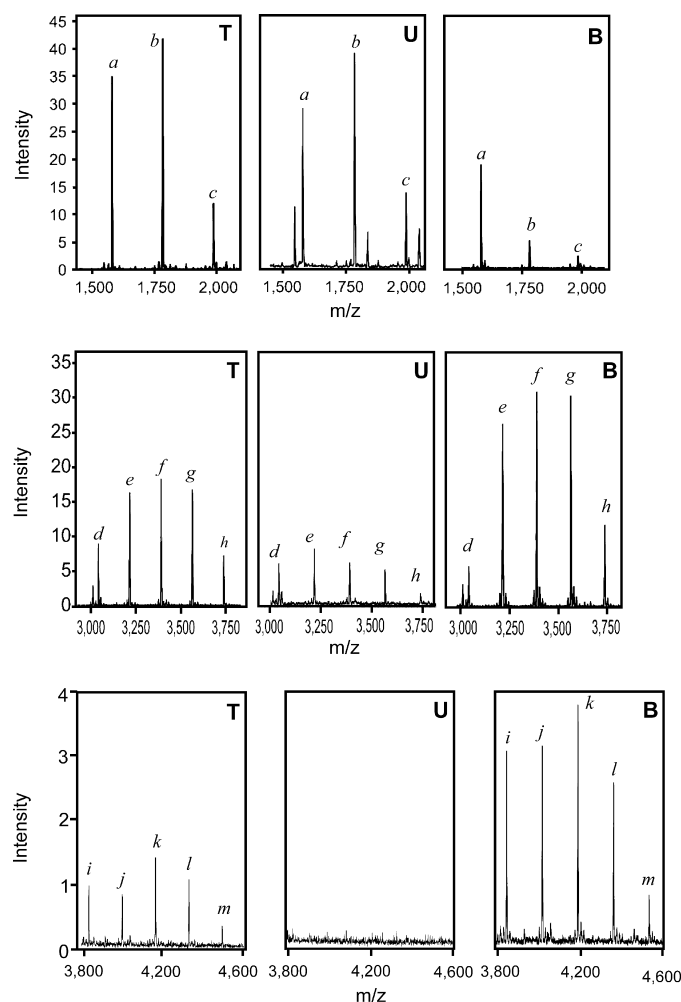


FIG. 4. Comparison of the MALDI spectra of different classes of N-glycans associated with fractions obtained by the chromatography of whole human seminal plasma on *Lotus* lectin-agarose. Spectra for the N-glycans in the total (T), unbound (U) and bound seminal plasma fractions (B) obtained following lectin affinity chromatography are arranged side by side for evaluation. Signals for the high mannose type N-glycans with the formula  $\text{Man}_{5-7}\text{GlcNAc}_2$  are indicated by notations a-c, respectively, in the top panel. In the middle panel, signals for the triantennary N-glycans bearing 2–6 fucose residues on their outer antennae are indicated by notations d-h, respectively. Tetraantennary N-glycans bearing 4–8 fucose residues in their outer antennae are indicated by the notations i-m, respectively, in the lower panel.

depletion was observed following affinity chromatography of two other samples of seminal plasma obtained from other donors (supplemental Figs. S1 and S2). These observations clearly indicated that affinity separation on *Lotus* lectin-agarose selected for seminal plasma glycoproteins that express triantennary and tetraantennary N-glycans bearing multiple terminal Lewis<sup>x</sup> and Lewis<sup>y</sup> sequences.

**Identification of the Major Seminal Plasma Glycoproteins Bound to *Lotus* Lectin-Agarose**—Specific bound and unbound fractions obtained during affinity chromatography on *Lotus* lectin-agarose shown in Fig. 2 were also subjected to SDS-PAGE gel analysis (Fig. 1). Electrophoretic analysis of the proteins in fraction 1 yielded the same profile that was observed in whole seminal plasma. The bound fractions from *Lotus* lectin affinity chromatography obtained from the separation of three seminal plasma samples were pooled and

subjected to SDS gel electrophoresis (Fig. 5). Major bands were detected at 190 kDa, 85–90 kDa, 55 kDa and 35–40 kDa. Proteomic analysis of the proteins indicated that the 190 kDa band was a mixture of angiotensin-converting enzyme and a small amount of seminogelin-I. Both of these proteins are not glycosylated and are therefore not ligands for DC-SIGN. Upon further inspection, the 85–90 kDa band was found to consist of two very closely spaced bands, which were isolated and separately analyzed. The upper band migrating nearer 90 kDa was determined to be clusterin. The lower band migrating nearer 85 kDa was identified as the galectin-3 binding protein (Mac-1) with some contaminating clusterin. The band at 55–60 kDa consisted of protein C inhibitor (PCI). The broad band at 35–40 kDa was identified as prostatic acid phosphatase (PAP). Details on the identification of these glycoproteins are reported in supplemental Table S1.

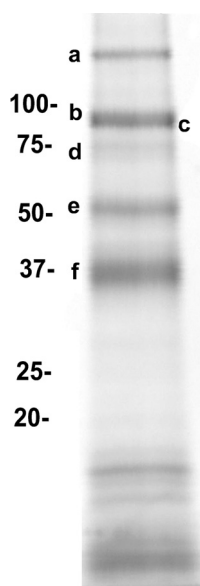


FIG. 5. **SDS gel electrophoretic sizing of the major seminal plasma glycoproteins bound to *Lotus* lectin-agarose.** The bound glycoproteins eluted with 0.1 M fucose containing buffer obtained from three different samples of human seminal plasma were pooled and concentrated down to a final volume of 1 ml. A 20- $\mu$ l aliquot of this fraction was subjected to SDS-gel electrophoresis and stained with colloidal Coomassie blue stain as discussed in the Methods. Individual bands were cut from this gel, and subjected to proteomic analysis as outlined in the Methods.

**DC-SIGN Binding to Seminal Plasma Glycoproteins Separated on *Lotus* Lectin-Agarose**—There was very little binding of DC-SIGN to fraction 1 that eluted without retention from *Lotus* lectin-agarose (Fig. 1). This result confirmed that the weak DC-SIGN reactive bands present in the total seminal plasma fraction were substantially depleted by this lectin affinity separation. However, there was very strong binding of DC-SIGN to the glycoproteins in fraction 11 that were retained by *Lotus* lectin-agarose (Fig. 1). Four distinct DC-SIGN reactive bands were identified, consistent with bands comigrating with clusterin, galectin 3-binding protein, protein C inhibitor and PAP. DC-SIGN also reacted directly with all four of these glycoproteins in solution, as indicated in immunoprecipitation studies shown in Fig. 6. These results confirmed that affinity chromatography on *Lotus* lectin-agarose was very useful for enriching for the endogenous glycoprotein ligands of DC-SIGN in human seminal plasma.

#### DISCUSSION

Proteomic analysis previously confirmed the presence of 923 proteins in human seminal plasma (30). After ejaculation, some proteins are rapidly degraded by PSA, a very abundant serine protease in seminal plasma also known as  $\gamma$ -seminoprotein, kallikrein-3 or semenogelase (31). This proteolysis leads to a very substantial loss of viscosity of human semen. A previous glycomics analysis of whole seminal plasma indicates that there are three major classes of N-glycans associ-

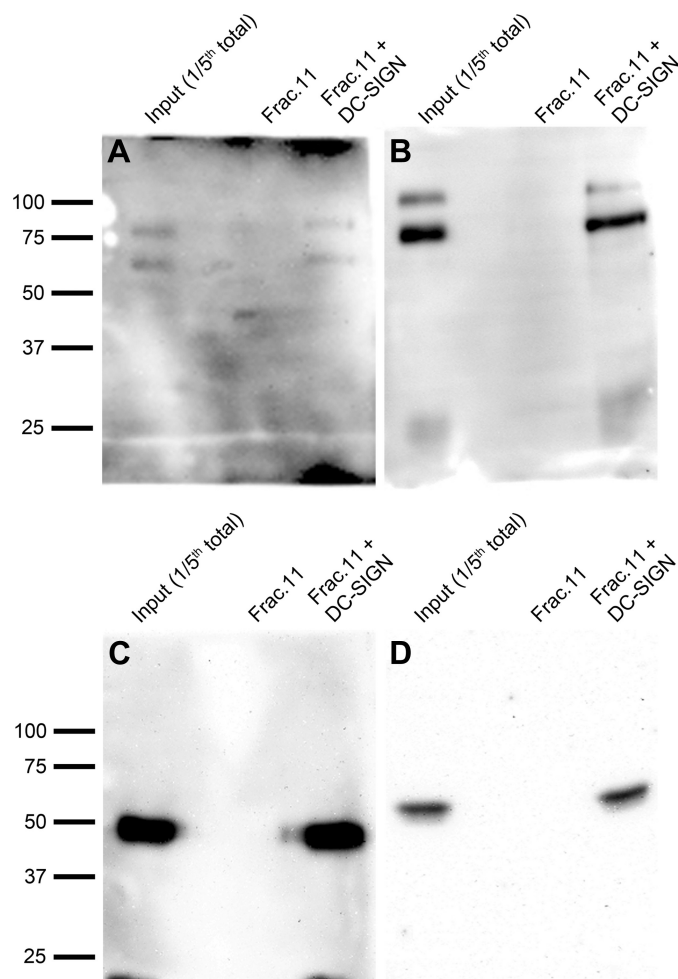


FIG. 6. **DC-SIGN binds to specific seminal plasma glycoproteins.** Seminal plasma proteins eluted from the *Lotus* lectin affinity column (Fraction 11) were incubated with protein A-Sepharose beads either alone or in the presence of DC-SIGN-Fc. Proteins bound to beads were analyzed by Western blot with antibodies against clusterin (panel A), galectin 3-binding protein (panel B), prostatic acid phosphatase (panel C) or protein C inhibitor (panel D). Input corresponds to 1/5<sup>th</sup> of the total material present in each reaction, while the Frac. 11 lanes display the background staining contributed by non-specific binding to the protein A-Sepharose beads.

ated with glycoproteins in this fluid: (1) bi-, tri-, and tetra-antennary core-fucosylated complex type N-glycans with antennae terminated with multiple Lewis<sup>x</sup> and/or Lewis<sup>y</sup> sequences; (2) high mannose type N-glycans (Man<sub>5-7</sub>GlcNAc<sub>2</sub>); and (3) bi-, tri-, and tetra-antennary core-fucosylated complex type N-glycans bearing antennae capped with either sialic acid or galactose (17). The most abundant class of N-glycans is terminated with Lewis<sup>x</sup> and/or Lewis<sup>y</sup> sequences (Fig. 3A). Similar types of N-glycans are also associated with purified human spermatozoa (17). The representation of sialylated N-glycans is highly variable between fertile human males, with some individuals expressing substantial amounts of these oligosaccharides and others only very minor amounts of sialylated glycoproteins (17).



TABLE I  
Carbohydrate Ligands for DC-SIGN

Designation	Structure*	Sequence
Lewis <sup>a</sup>		Galβ1-3GlcNAc 4 Fucα1
Lewis <sup>b</sup>		Fucα1-2Galβ1-3GlcNAc 4 Fucα1
Lewis <sup>x</sup>		Galβ1-4GlcNAc 3 Fucα1
Lewis <sup>y</sup>		Fucα1-2Galβ1-4GlcNAc 3 Fucα1
Sulfo-Lewis <sup>a</sup>		SO <sub>3</sub> <sup>-</sup> -3Galβ1-3GlcNAc 4 Fucα1
Pseudo-Lewis <sup>y</sup>		Fucα1-3Galβ1-4GlcNAc 3 Fucα1

\*Key to Symbols: Gal GlcNAc Fuc Sulfate

It is apparent from this study that the amount of endogenous glycoprotein ligand for DC-SIGN is relatively low in seminal plasma glycoproteins, despite the very substantial expression of terminal Lewis type sequences on these glycoproteins (Fig. 3). Affinity chromatography of the seminal plasma fraction on *Lotus* lectin-agarose was employed to select for endogenous glycoprotein ligands for this immune lectin. This approach was previously used in another investigation to select for fucosylated glycoproteins (32). However, no subsequent glycomic analyses were performed to confirm the enrichment of glycans that react with *Lotus* lectin in the bound glycoprotein fraction. In this investigation, glycoproteins bound to this matrix were clearly shown to express more triantennary and tetraantennary N-glycans terminated with multiple Lewis<sup>x</sup> and Lewis<sup>y</sup> sequences.

DC-SIGN binds to both high mannose type N-glycans and terminal Lewis type sequences (Table I) (4, 33). The glycoprotein ligands for DC-SIGN in seminal plasma were preferentially bound to *Lotus* lectin-agarose. The unbound seminal plasma glycoproteins obtained during this separation display substantial levels of high mannose type N-glycans compared with the bound fraction (Fig. 4 (top panel)). These results indicate that the high mannose type N-glycans on seminal plasma glycoproteins are not presented in a sufficient valency

or steric presentation on seminal plasma glycoproteins to enable interaction with DC-SIGN.

Proteomic analysis of the fraction bound to *Lotus* lectin-agarose indicated the presence of four major endogenous glycoprotein ligands for DC-SIGN. One of these glycoproteins is clusterin, also known as sulfated glycoprotein-2 (34), apolipoprotein J (35), complement-associated protein (SP-40,40) (36), complement cytotoxicity inhibitor (37), testosterone-repressed prostate message 2 (38), and Ku70 binding protein-1 (39). Clusterin is universally present in all human body fluids (40). However, the concentration of this glycoprotein in human seminal plasma is about fourfold higher ( $438 \pm 285 \mu\text{g/ml}$ ) than it is in human serum ( $111 \pm 50 \mu\text{g/ml}$ ) (41). Clusterin glycoforms in the circulation express highly sialylated complex type N-glycans, but no Lewis antigens or O-glycans (42). Clusterin is unusual because its expression is either increased or decreased relative to normal serum levels in many human cancers including malignancies originating in the bladder (43), breast (44), colon (45), kidney (46), liver (47), ovary (48), pancreas (49), and prostate (50). For these reasons, clusterin is considered to be a major tumor biomarker glycoprotein.

Another major glycoprotein in the *Lotus* lectin bound fraction is galectin-3 binding protein, also known as lectin galactoside-binding soluble 3-binding protein (51), Mac-2-binding protein (52), tumor-associated antigen 90K (53), and basement membrane autoantigen p105 (54). Galectin-3 binding protein is a very minor constituent of human serum ( $8.8 \pm 3.9 \mu\text{g/ml}$ ) (55). However, elevated levels of this glycoprotein are observed in the serum of patients with breast, colon, neuroendocrine, ovarian, non-small-cell-lung, colon, oral, prostate tumors and lymphomas (including non-Hodgkin's lymphomas) (56).

Perhaps the best known tumor marker present in the *Lotus* lectin bound fraction is PAP. This phosphatase is an extremely abundant glycoprotein in human seminal plasma ( $1.26 \text{ mg/ml}$ ) (57). Recent structural analyses of the seminal plasma glycoforms indicate that this glycoprotein is N-glycosylated but not O-glycosylated (58). In 1941, Huggins and Hodges detected elevated levels of PAP in the serum of males with prostate cancer by using an assay based on enzymatic activity (59). PAP became the first tumor biomarker used for staging any form of cancer. Testing for serum PAP levels is still employed by urologists to predict outcomes. Patients with elevated serum levels of PAP have only a 39% 4-year disease-free survival *versus* 79% for patients with normal serum levels (60).

The only other major endogenous ligand for DC-SIGN in seminal plasma that has not been previously associated with oncogenesis is PCI. This 55–60 kDa glycoprotein is a serine protease inhibitor (serpin) that blocks the activity of thrombin, active protein C, factor Xa, factor XIa, plasma and tissue kallikreins, urokinase and tissue plasminogens (61–64). The level of this glycoprotein in seminal plasma is about  $160 \pm 20 \mu\text{g/ml}$ , or about 30- to 40-fold higher than in human plasma



(65). PCI rapidly inactivates PSA after ejaculation (66). Complexes of PCI and PSA are present in seminal plasma, consistent with this proposed physiological role (65). However, such complexes were not detected in the fractions bound to *Lotus* lectin-agarose. Recently, PCI was immunopurified from seminal plasma and its oligosaccharides subjected to structural analysis (66). The seminal plasma glycoforms of this glycoprotein expressed: (1) very low levels of high mannose-type oligosaccharides; (2) virtually no sialylated antennae; (3) an extremely high density of terminal Lewis<sup>x</sup> and Lewis<sup>y</sup> sequences on its complex type N-glycans; and (4) no O-glycans. The current observation that PCI is retained on *Lotus* lectin-agarose and reacts with DC-SIGN is consistent with the results of this structural analysis.

Endogenous glycoprotein ligands for DC-SIGN have been proposed as playing a major role in maintaining immune homeostasis (1). However, the exact molecular requirements for the interaction of such ligands with DC-SIGN have not been defined. As noted in this study, glycomic analyses indicated that glycoproteins expressing tri- and tetraantennary N-glycans bearing multiple Lewis<sup>x</sup> and/or Lewis<sup>y</sup> sequences were readily bound to immobilized *Lotus* lectin and also to DC-SIGN during Western blot analysis and immunoprecipitation studies.

These endogenous ligands from seminal plasma also share another feature that could play a role in their binding to DC-SIGN. Clusterin isolated from human serum bears a total of six occupied N-glycosylation sites associated with its disulfide-linked  $\alpha$ -chain (Asn<sup>64</sup>, Asn<sup>81</sup>, Asn<sup>123</sup>) and  $\beta$ -chain (Asn<sup>64</sup>, Asn<sup>127</sup>, Asn<sup>147</sup>) (42). Evidence was also obtained for the occupancy of six of the seven potential N-glycosylation sites (Asn<sup>69</sup>, Asn<sup>125</sup>, Asn<sup>192</sup>, Asn<sup>398</sup>, Asn<sup>551</sup>, Asn<sup>580</sup>) in galectin-3 binding protein (67). Protein C inhibitor obtained from human serum is usually N-glycosylated at three sites (Asn<sup>230</sup>, Asn<sup>243</sup>, and Asn<sup>319</sup>) (68). PAP is also N-glycosylated at three sites (Asn<sup>62</sup>, Asn<sup>188</sup>, Asn<sup>301</sup>) (69, 70). In summary, all of the major endogenous glycoprotein ligands for DC-SIGN detected in human seminal plasma express multiple N-glycosylation sites. The presentation of Lewis<sup>x</sup> and/or Lewis<sup>y</sup> sequences on more than one N-glycan could also be essential for DC-SIGN binding, though more studies must be performed to confirm this requirement.

The physiological role of this very unusual addition of terminal Lewis<sup>x</sup> and Lewis<sup>y</sup> sequences to glycoconjugates in the urogenital tract of the human male has not yet been determined. However, studies performed on the gastric pathogen *Helicobacter pylori* confirm that isogenic variants that express Lewis<sup>x</sup> and Lewis<sup>y</sup> sequences on their lipopolysaccharides induce tolerance in the human gastric mucosa (71–73). This deviation of the human immune response is associated with specific shifts in cytokine expression by naïve dendritic cells (DCs) and T cell polarization from a Th1 toward a Th2 response (70). These effects are dependent on signaling mediated by the interaction of these lipopolysaccharides with DC-

SIGN (71, 74). By contrast, infection with isogenic variants of *H. pylori* that lack terminal Lewis<sup>x</sup> and Lewis<sup>y</sup> sequences induces the very severe pathological effects that are classically associated with a Th1 type response (71).

Such tolerizing effects could be very useful in the human male urogenital tract. It is well established that autoantigens (neoantigens) are produced in this system during puberty, well after the period of thymic education that defines tolerance to self (75, 76). Proof for the existence of such testicular autoantigens is the development of experimental autoimmune orchitis following the injection of autologous or syngeneic testicular homogenates at sites outside of the testes in mice and humans (77, 78). The expression of these endogenous ligands for DC-SIGN in the male urogenital tract during the onset of puberty could contribute to the development of immune homeostasis that prevents autoimmune reactions.

The Lewis<sup>y</sup> sequence was initially detected as a tumor associated carbohydrate antigen (79). Subsequent probing of normal and neoplastic cells with anti-Lewis<sup>y</sup> monoclonal antibodies revealed that this epitope is highly expressed on >70% of all human tumors of epithelial origin, but not on normal cells and tissues located outside of the human male urogenital tract (80–90). The expression of Lewis<sup>y</sup> sequences on human tumor cells is usually inversely correlated with survival time (90). Data obtained in this study have revealed that three of the four major endogenous glycoprotein ligands for DC-SIGN in human seminal plasma are also tumor biomarker glycoproteins. Whether these same glycoproteins preferentially acquire the Lewis<sup>y</sup> sequence in tumor cells is currently unknown.

Schistosomes also express a very close structural analog of the Lewis<sup>y</sup> sequence on their surface, known as the pseudo-Lewis<sup>y</sup> antigen (Table I). This unusual Lewis analog is also a potent carbohydrate ligand for DC-SIGN (91). Thus, it is quite possible that schistosomes, *H. pylori*, and aggressive tumor cells engage the same pathway that is used to abrogate immune responses directed against autoantigens that are initially expressed in the male urogenital tract following the onset of puberty. This linkage could represent yet another pathway of physiological immune deviation that pathogens and aggressive tumor cells exploit to ensure their survival.

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